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Research Article

Mixed "Antiandrogenic" Chemicals at Low Individual Doses Produce Reproductive Tract Malformations in the Male Rat

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ABSTRACT

Biomonitoring efforts have clearly shown that all humans are exposed to chemical mixtures. Of concern is whether or not exposure to mixtures during pregnancy contributes to congenital abnormalities in children even when each chemical is at an individual dose that does not affect the fetus. Here, we hypothesized that *in utero* exposure to a mixture of chemicals covering multiple "antiandrogenic" mechanisms of action at doses that individually have no adverse effect would result in permanent reproductive tract alterations in the male rat after birth. Pregnant dams were exposed to a range of dilutions (100%, 50%, 25%, 12.5%, 6.25%, or vehicle control) of a mixture containing pesticides, phthalates, and drugs (p, p'-DDE, linuron, prochloraz, procymidone, pyrifluquinazon, vinclozolin, finasteride, flutamide, simvastatin, and 9 phthalates [dipentyl, dicyclohexyl, di-2-ethylhexyl, dibutyl, benzyl butyl, diisobutyl, diisoheptyl, dihexyl, and diheptyl]). The top dose contained each chemical at 20% of its lowest observed adverse effect level (LOAEL) for the most sensitive male reproductive alteration following *in utero* exposure. We found that male rat offspring displayed a variety of neonatal, pubertal, and permanent adult effects across all dose levels. Even at the lowest dose (each chemical approximately 80-fold below lowest observed adverse effect level) there were permanent reductions in several reproductive tract tissue weights. In the top dose group, 100% of male offspring displayed permanent severe birth defects including genital malformations. Despite acting via 5 different molecular initiating events, a mixture of 18 chemicals can combine to produce additive effects even when each compound is at is at a relatively low dose.

Key words: chemical mixtures; dose addition; birth defect; phthalate; pesticide; male reproductive tract.

Modern industrial reliance on synthetic chemicals has resulted in people being exposed to complex chemical mixtures (CDC, 2009). The overriding presumption has been that exposure to very low doses (ie, below experimentally determined effect levels) of any individual chemical carries little risk of adverse effects. This is partially due to a general lack of data demonstrating whether exposure to mixtures of toxic chemicals, such as endocrine disrupting compounds, actually produce cumulative, adverse effects at exposure levels below which the individual chemicals have been shown to produce serious effects alone (Kortenkamp *et al.*, 2007). We contend that the greater the number of chemicals present in a mixture that operate via a similar biological pathway or have the same target tissues, the lower the dose of any individual chemical in the mixture that is required for contributing towards cumulative effects.

Chemical mixture toxicity is particularly important when considering *in utero* exposures due to the high sensitivity of the embryo and fetus to developmental perturbations. Notably, 2 of the most commonly occurring human birth defects are hypospadias of the male phallus (approximately 0.3%–0.8% of live births [Carmichael *et al.*, 2012; Mai *et al.*, 2015]) and cryptorchidism of the testes (approximately 1%–3% of live births [Thonneau *et al.*, 2003]), with some studies indicating that the incidence rates for both have increased during the last 30–40 years (Paulozzi, 1999; Toppari *et al.*, 2001). Over a similar time period, other studies have reported declines in male sperm production leading to reduced fertility (Levine *et al.*, 2017) and increases in testicular germ cell cancers (Nigam *et al.*, 2015; Skakkebaek, 2017). Exposure to environmental chemicals that alter androgen signaling during fetal development have been implicated in these human health trends (termed Testicular Dysgenesis Syndrome; Skakkebaek *et al.*, 2016) because *in utero* animal studies have been able to recapitulate many of the congenital reproductive abnormalities observed in humans.

Development and maintenance of male reproductive tissues is critically dependent on androgen signaling. Chemicals can disrupt androgen signaling in the male fetus through several different molecular initiating events (MIEs) including antagonizing the androgen receptor (AR) and/or impairing androgen synthesis in androgen-dependent tissues (Rider *et al.*, 2009; Wilson *et al.*, 2008). For example, the pharmaceutical finasteride inhibits 5α-reductase preventing conversion of testosterone to dihydrotestosterone (Clark *et al.*, 1990) and the cholesterol-lowering agent simvastatin inhibits 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, preventing endogenous synthesis of cholesterol (a testosterone precursor) (Beverly *et al.*, 2014). The herbicide linuron (used on a variety of vegetable crops) both inhibits cytochrome P450 enzymes that are critical for *de novo* synthesis of testosterone in the testis (Wilson *et al.*, 2009) and antagonizes the AR (Lambright *et al.*, 2000; McIntyre *et al.*, 2000), whereas the fungicide vinclozolin (used in landscaping, greenhouses, and plant nurseries) is a classic AR antagonist, but does not affect testosterone production (Gray *et al.*, 1994). Further, the specific MIE(s) for the entire phthalate ester chemical class (nearly ubiquitous compounds found in plastics, cosmetics, personal care products, and other consumer items) is/are unknown, however extensive research has demonstrated dramatic reductions in fetal rat testis testosterone production and insulin-like hormone 3 (Insl3, critical for testis descent) following *in utero* exposure (Parks *et al.*, 2000; Wilson *et al.*, 2004). Despite a multiplicity of chemical mechanisms, each of these compounds is "antiandrogenic" and can impair androgen signaling in fetal male reproductive tissues, ultimately producing birth defects, decreased adult reproductive capacity, and/or lead to neoplastic lesions (Figure 1) .

Building on previous mixture studies that demonstrated cumulative effects of "antiandrogenic" chemicals (Christiansen *et al.*, 2009; Hotchkiss *et al.*, 2004, 2010; Rider *et al.*, 2008, 2010), here we asked whether adverse effects occur if many "antiandrogenic" chemicals are combined at doses below the lowest levels previously shown to elicit adverse effects individually. Pregnant dams were dosed orally with a mixture of 18 different "antiandrogenic" chemicals during the window of gestation in which male reproductive tract development is most sensitive to chemical insult. For each chemical, we identified the lowest lowest observed adverse effect level (LOAEL) from the literature for *any* adverse effect on development of the male reproductive system (Table 1). The top dose contained each chemical at 20% of its respective LOAEL (ie, LOAEL/5) followed by a 50% dilution series down to each chemical at LOAEL/80. The mixture was tested in both an assay for investigating fetal testis testosterone production and testis gene expression, as well as an assay for evaluating early postnatal malformations and permanent adverse effects in mature adults following *in utero* exposure. We hypothesized that these chemicals would act cumulatively to produce adverse effects at doses below which any individual chemical would be expected to produce an effect alone, despite the fact that they have different mechanisms of toxicity and therefore would not currently be grouped for cumulative risk assessment (USEPA, 2002).

MATERIALS AND METHODS

Chemicals and dosing. Dosing solutions were prepared using laboratory-grade corn oil (Sigma-Aldrich, St. Louis, Missouri). Vinclozolin (CAS 50471-44-8) was purchased from Riedel-de Haen (Seelze, Germany). Linuron (CAS 330-55-2) was purchased from Crescent Chemical Company (Islandia, New York). Procymidone (CAS 32809-16-8) and Pyrifluquinazon (CAS 337458-27-2) were purchased from Chem Services (West Chester, Pennsylvania). Flutamide (CAS 13311-84-7), Prochloraz (CAS 067747-09-5), Dicyclohexyl phthalate (CAS 84-61-7), diisoheptyl phthalate (CAS 71888-89-6), and p, p'-DDE (CAS 72-55-9) were purchased from Sigma-Aldrich. Simvastatin (CAS 79902-63-9) was purchased from AK Scientific (Union City, California). Finasteride (CAS 98319-26-7) was purchased from Merck (West Chester, Pennsylvania). Dipentyl phthalate (CAS 131-18-0), diethylhexyl phthalate (CAS 117-81-7), dibutyl phthalate (CAS 84-74-2), butyl benzyl phthalate (CAS 85-68-7), diisobutyl phthalate (CAS 84-69-5), dihexyl phthalate (CAS 84-75-3), and diheptyl phthalate (CAS 3648-21-3) were gifted from the National Toxicology Program (NTP) (Research Triangle Park, North Carolina). All chemicals were ≥98% purity as verified by the manufacturer and phthalates from NTP were independently verified for purity (>99%) by Research Triangle Institute (Durham, North Carolina).

Treatments were prepared as a fixed ratio dilution series based on the individual chemical LOAELs (Table 1 and Supplementary Table 1). For each chemical we selected the lowest LOAEL for any effect on male reproductive tissues from studies that were conducted on pregnant rats with oral dosing that included the masculinizing window of gestation (GD14-18). The top dose (100% dose) contained each chemical at 1/5th of its conservative LOAEL for male reproductive effects, followed by a 50% dilution series down to each chemical at 1/80th of the LOAEL (5 total doses, 100, 50, 25, 12.5, and 6.25% corresponding to 1/5, 1/10, 1/20, 1/40, and 1/80th of the LOAEL). Pregnant dams were dosed via oral gavage with either vehicle control or treatment dilution at 2.5 ml kg^{-1} from GD14-18.

Animals. Time-mated female Sprague Dawley rats (Crl: CD[SD]), approximately 90-days old, were purchased from Charles River Laboratories (Raleigh, North Carolina) and shipped to USEPA (Research Triangle Park, North Carolina) on gestation day 2 (GD2; day of confirmed mating = GD1). Pregnant dams were housed individually in clear polycarbonate cages (20 × 25 × 47 cm) with heat-treated, laboratory-grade pine shavings and fed NIH07 rodent diet and filtered (5 μm) municipal tap water *ad libitum*. Dams were weight-ranked to produce similar mean weights and randomly assigned to treatment groups. This study was conducted in accordance with a protocol approved by the USEPA National Health and Environmental Effects Research Laboratory's Institutional Animal Care and Use Committee.

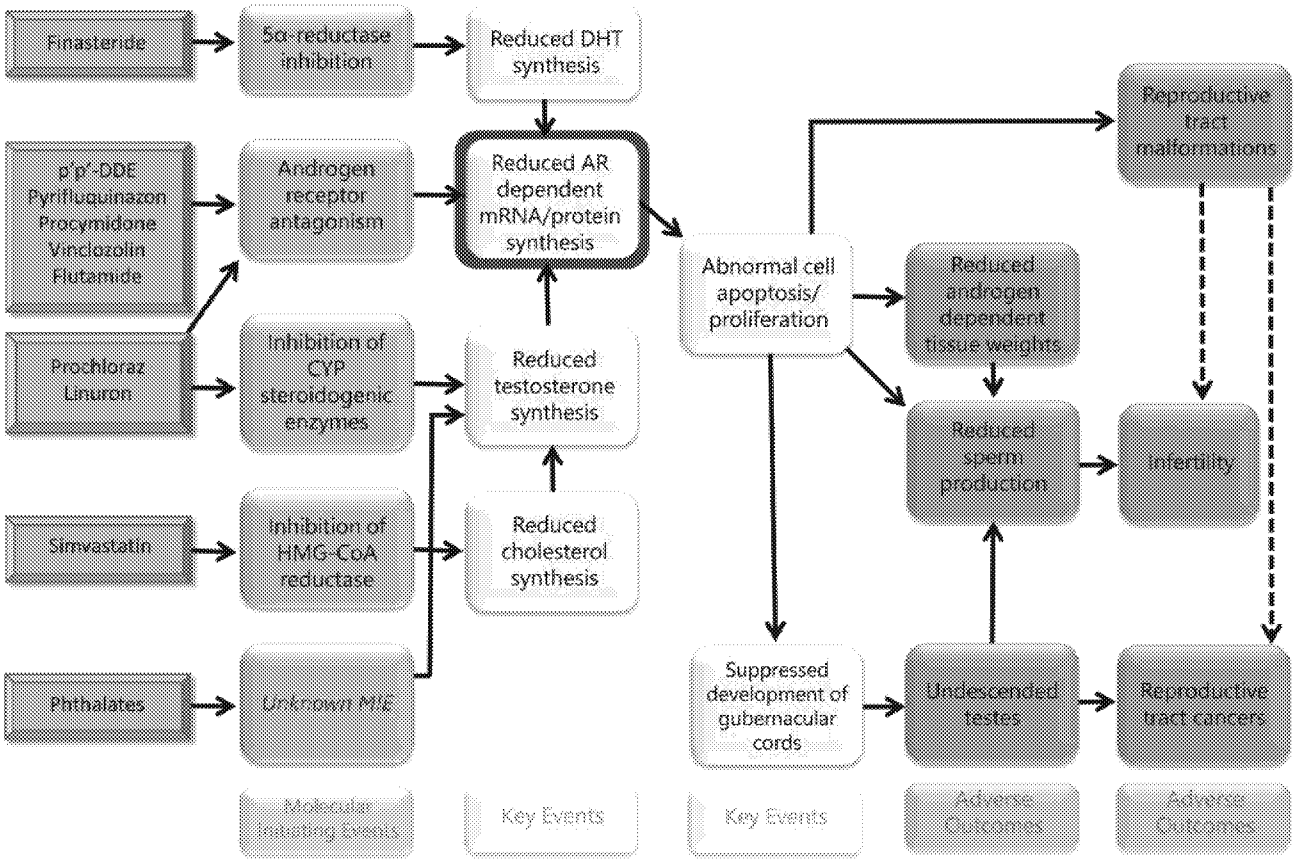

Figure 1. AOP network for chemicals that disrupt AR-mediated cellular signaling leading to adverse effects on development of the male reproductive tract resulting from *in utero* exposure. The bold outlined KE indicates the critical node that links the various MIEs to the downstream AOs. Abbreviations: DHT, dihydrotestosterone; AR, androgen receptor; CYP, cytochrome P450; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A.

Table 1. Individual Chemical LOAELs for Any Effect on Development of Male Reproductive Tract Following *In Utero* Exposure Identified for Basis of Chemical Mixture Dosing

Chemical	LOAEL (mg kg^{-1} day^{-1})	Effect[a]	100% Dose (mg kg^{-1} day^{-1})
Finasteride	0.03	AGD (Clark *et al.*, 1993)	0.006
Flutamide	2.5	AGD (Fussell *et al.*, 2015; Miyata *et al.*, 2002)	0.5
Vinclozolin	6	NR, AST (Hellwig *et al.*, 2000)	1.2
Diethylhexyl ph.	11	RTM (Gray *et al.*, 2009)	2.2
Linuron	12.5	NR (McIntyre *et al.*, 2000)	2.5
Procymidone	25	NR, AGD, AST (Ostby *et al.*, 1999)	5.0
Pyrifluquinazon	25	AGD, NR, RTM[b]	5.0
Prochloraz	31.3	FTP (Blystone *et al.*, 2007), NR (Noriega *et al.*, 2005)	7.5
Dipentyl ph.	33	FTP (Hannas *et al.*, 2011a), RTM (Gray *et al.*, 2016)	6.6
Simvastatin	62.5	FTP (Beverly *et al.*, 2014)	12.5
Dibutyl ph.	100	NR (Mylchreest *et al.*, 2000)	20
p, p'-DDE	100	AGD, NR (Kelce *et al.*, 1995)	20
Diisoheptyl ph.	227	AGD (McKee *et al.*, 2006)	45
Dicyclohexyl ph.	250	AGD (Saillenfait *et al.*, 2009a)	50
Butylbenzyl ph.	250	AGD (Tyl *et al.*, 2004)	50
Diisobutyl ph.	250	AGD, NR (Saillenfait *et al.*, 2008)	50
Dihexyl ph.	250	AGD (Saillenfait *et al.*, 2009b)	50
Diheptyl ph.	500	AGD (Saillenfait *et al.*, 2011)	100

The top dose level (100%) contained each chemical at LOAEL/5 (see Supplementary Table 1 for chemical concentrations across complete dosing structure).

[a]Reduction in anogenital distance (AGD); reduction in fetal testis testosterone production (FTP); increase in female-like nipple/areolae retention (NR); reduction in male reproductive accessory sex tissues (AST); reproductive tract malformations (RTM).

[b]Unpublished data from Laboratory of L.E.G., Jr. (USEPA).

Animals were housed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care and maintained at 20°–22°C, 45%–55% humidity, and a 12:12-h photoperiod (lights off 18:00).

Fetal testis screen. Specifics of this method have been previously reported in Furr *et al.* (2014). Briefly, 3 blocks of 15 dams per block were dosed via oral gavage with vehicle ($n = 11$ dams), 100% ($n = 6$), 50% ($n = 9$), 25% ($n = 6$), 12.5% ($n = 6$), or 6.25% ($n = 7$) treatments from GD14 to 18. Late gestation (GD18) dams were euthanized by decapitation and pups were removed via cesarean section. Fetal testes were collected from male pups with a single testis from the first 3 males used for determination of testosterone production and the remaining testes used for gene expression analysis. *Ex vivo* testosterone production was quantified using Coat-a-Count radioimmunoassay (Siemens Healthcare Diagnostics, Deerfield, Illinois) according to manufacturer specifications following 3 h incubation in M-199 media. Gene expression was assessed using reverse transcriptase real-time PCR of cDNA synthesized from RNA extracted from testis homogenates. A 96-well gene array plate was previously custom designed to contain 89 target genes and 3 housekeeping genes, an intra-assay control, a genomic DNA control, a reverse transcriptase control, and a positive PCR control (SABioscience, Frederick, Maryland; Hannas *et al.*, 2012). PCR reaction was run on an iCycler iQ Real-Time Detection System (BioRad, Hercules, California) using RT2 SYBR Green qPCR Master Mix (SABioscience).

Postnatal reproductive development. Specifics for this methodology have been previously reported in Gray *et al.* (2009). Briefly, $n = 5$ dams per treatment group and vehicle control were dosed via oral gavage from GD14–18. Dams were allowed to give birth naturally (approximately GD21) and on PND2 pups were sexed, weighed, and anogenital distance (AGD) was measured using a dissecting microscope with an ocular micrometer. On PND13 pups were again sexed, weighed, and nipple/areolae retention was visually scored. On PND21 dams were euthanized, uterine implantation sites were scored, pups were weaned to 2 animals per cage by sex and treatment group, and food was changed to NTP2000 rodent diet. Beginning on PND29 for female offspring and PND40 for male offspring, individuals were evaluated at the same time every other day for markers of pubertal onset, vaginal opening (VO) for females and balano-preputial separation (PPS) for males. Beginning at PND90 female offspring were considered to be mature adults and were weighed, euthanized via decapitation, and examined via necropsy for any reproductive tract abnormalities. Similarly, males reached maturity at PND120 and were weighed, euthanized, and examined for a range of reproductive tract malformations and weights were collected for all relevant reproductive tissues. Male necropsy included scoring nipple/areolae retention, external genital malformations (eg, hypospadias), internal malformations (eg, epididymal agenesis, testicular atrophy, undescended testes), and weights of glans penis, ventral prostate, seminal vesicles, testes, epididymides, levator ani-bulbocavernosus (LABC), and bulbourethral (Cowper's) glands. After weighing, the left epididymis was separated into 2 sections, the cauda and the corpus + caput, and individually minced in M-199 media. Total sperm counts in epididymal sections were measured using a Multisizer 3 coulter counter (Beckman Coulter, Brea, California). All remaining tissues were preserved in Bouin's fixative for 24 h, then transferred to 70% ethanol. Both testes and the right epididymis were shipped to Experimental Pathology Laboratories, Inc. (Durham, North Carolina) where tissues were embedded, sectioned, stained with hematoxylin and eosin, and evaluated by a Diplomate of the American College of Veterinary Pathology.

Statistics. All reported values are mean ± SEM and all statistical comparisons were conducted at $\alpha = 0.05$ significance level except for gene expression which utilized $\alpha = 0.01$ to detect significant ANOVA results. Data were analyzed using PROC MIXED in SAS (v.9.4, SAS Institute, Cary, North Carolina) in order to correct for the nested effects of individuals within litters. Pairwise comparison of significant ANOVA results was performed using the least squares means procedure in SAS. All data were analyzed using litter as the statistical unit. Fetal testis gene expression C_T values were converted to fold values using the equation $F = 2^{-\Delta\Delta C}{}_T$, followed by $\log_{10}$ transformation. Fetal testis testosterone production was normalized to the mean control concentration within a given block and analyzed as % of control values. Dose response analyses for fetal testis testosterone production, postnatal AGD, and nipple retention were conducted using 4-parameter logistic regression in GraphPad Prism v7.02 (GraphPad, Inc., La Jolla, California) with the following parameter constraints: bottom = 0%, top = 100%. Mean female AGD was subtracted from male AGD in order to calculate % reduction as compared to control.

RESULTS

Fetal Testis Gene Expression and Testosterone Production

Significant reductions in expression of genes in the fetal testis related to testosterone synthesis (*Cyp17a1*), cholesterol uptake and transport (*Scarb1*, *StAR*), conversion of cholesterol to pregnenolone (*Cyp11a1*), and the adrenal enzymes *Cyp11b1* and *Cyp11b2* occurred in the 50% and 100% dose groups (Figure 2). Expression levels of *Insl3* (critical for testis descent) and *Dhcr7* (critical for cholesterol synthesis) were significantly reduced in the 100% dose group. Interestingly, the testosterone synthesis related gene, *Hsd17b3*, commonly down-regulated with anti-androgen exposure, was significantly up-regulated in the 50% and 100% doses. Several other genes were significantly upregulated in the 100% dose, including *Rara* (retinoic acid signaling), *Ppard* (peroxisome proliferation), *Sra1* (varied nuclear receptor activity), *Gata4* (testicular development), and *Sox9* (regulation of antiMuellerian hormone). *Rhox5* (reproductive transcription factor) and *Ntf3* (related to neuron survival) were upregulated in the 50% and 100% doses, while *Lhx1* (development of renal and urogenital system), *Esr1* and *Esr2* (estrogen signaling) were upregulated in the 25%–100% doses (*Esr1* was not significant in 100% dose). The most sensitive genes were *Hsd3b* (testosterone synthesis enzyme) and *Sfrp2* (modulation of Wnt signaling), which were significantly up- and down-regulated in the 12.5% dose group, respectively.

Fetal testis testosterone production was reduced in all dose groups, with significant reductions in the 50% (53% ± 4% reduction) and 100% (69% ± 2% reduction) dose groups compared with controls (Figure 3, Table 2, Supplementary Table 2). Dose response regression estimated a 50% reduction in fetal testis testosterone production (ED_{50}) at 54.8% of the top dose (95% CI: 43.4%–72.7%; slope −1.71; $r^2 = 0.63$). During exposure, pregnant dams in the 100% dose group gained significantly less weight than controls (17.6 ± 2.5 vs 35.9 ± 2.7 g), however across all treatments there were no differences in the number per litter of

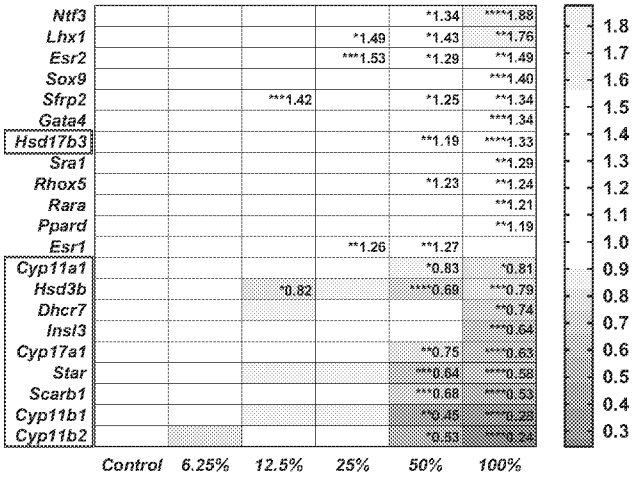

Figure 2. Heat map of differentially expressed genes in the fetal rat testis following *in utero* exposure to an 18-chemical mixture from GD14 to 18. Asterisks indicate level of significance (****$p < .0001$, ***$p < .001$, **$p < .01$, *$p < .05$) and listed values represent fold induction relative to control. Genes outlined in red have previously been identified to be affected by gestational phthalate exposure.

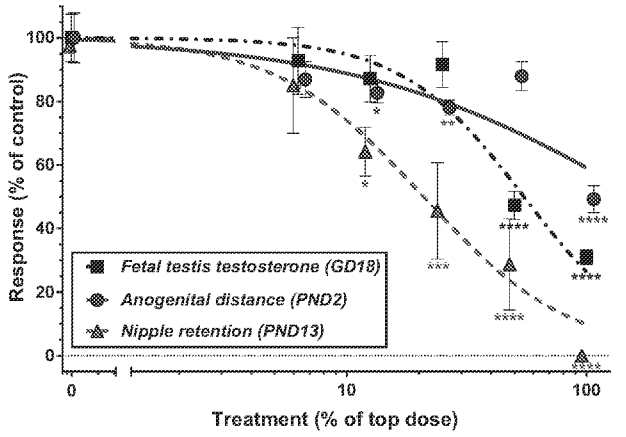

Figure 3. Dose response curves for *ex vivo* fetal testis testosterone production on GD18, AGD on PND2, and female-like nipple/areolae retention on PND13 in male offspring. Nipple retention presented as percent of males unaffected (ie, percent of males lacking retained nipples). Asterisks below data points indicate significant difference from controls (****$p < .0001$, ***$p < .001$, **$p < .01$, *$p < .05$).

total fetuses, live fetuses, fetal resorptions, or mean dam weight at assay termination (GD18).

Early-Life and Pubertal Effects

Male offspring displayed early life stage, dose-responsive defects in androgen-dependent tissue development. Mean AGD was reduced on postnatal day 2 (PND2) in male pups in all dose groups and was significant in the 12.5%, 25%, and 100% doses (Figure 3, Table 2, and Supplementary Table 2). Nonlinear regression of dose response estimated an ED_{50} of 164.6% of the top dose (92.5%–926%; slope −0.74; $r^2 = 0.50$). A similar dose-responsive increase in female-like nipple/areola retention occurred on PND13 in male offspring (Figure 3). All dose groups displayed multiple male pups with ≥ 1 retained nipple, while controls displayed only a single male pup with a single retained nipple. The percentage of male pups per litter with retained nipples was significantly greater than controls in the 12.5%, 25%, 50%, and 100% treatment groups (36%, 54%, 71%, and 100% incidence, respectively). Dose response regression estimated an ED_{50} of 20.8% of the top dose (14.0%–30.4%; slope 1.41; $r^2 = 0.68$) indicating this was the most sensitive early life stage endpoint. The number of nipples per male also increased with dose and was significant in the 25%–100% dose groups with means of 2.2, 2.2, and 9.2 nipples per male, respectively, compared with 0.03 nipples per male in the controls (Table 2 and Supplementary Table 2). There was no effect of treatment on female AGD, female nipple retention, or male/female body weight on PND2 or PND13.

In utero mixture exposure also delayed the onset of male puberty as measured by balano-PPS (Table 2 and Supplementary Table 2). Males in the 100% treatment group were significantly delayed (47.0 ± 0.6 days; $p = .0007$) in reaching reproductive maturity compared with controls (43.4 ± 0.3 days). Further, males in the 100% treatment group were significantly larger (270.1 ± 9.6 g; $p = .02$) than controls (230.0 ± 4.7 g), most likely as a result of the delay in puberty. There was no effect of treatment on timing of VO (a marker of pubertal onset) or body weight in females.

Permanent/Adult Postnatal Male Effects

Nearly all androgen-dependent male reproductive tissues displayed significant weight reductions as a result of *in utero* exposure to the 18 chemical mixture. Mean weights of all sex accessory tissues were lower than control in all treatment groups with many tissues displaying significant reductions at multiple doses (Table 2 and Supplementary Table 2). The most sensitive endpoint was LABC weight, which displayed significant reductions in all treatment groups including a 14% reduction at the lowest dose (Figure 4F). Paired testis weight and paired epididymis weight were both significantly lower than control in the 6.25%, 25%, and 100% treatment groups (tissue weights were also reduced in the 12.5% and 50% treatments at $p \leq .06$) (Figs. 4D and 4E). Paired seminal vesicle weight was significantly reduced at 25%, 50%, and 100% (6.25% and 12.5% groups reduced at $p \leq .06$). Glans penis weight was significantly reduced in the 12.5% and 100% treatment groups (Figure 4C). Ventral prostate weight was significantly reduced only at the 100% dose. The only tissue weight that did not differ from controls at any dose was the paired bulbourethral (Cowper's) glands. Adult male body weight at necropsy tended to decrease with increasing dose, however this effect was not significant ($p = .226$).

Permanent malformations of the external genitalia and internal sex accessory tissues largely occurred in the 100% dose group. The percentage of adult males with permanently retained female-like nipples/areolae was significantly elevated in the 25%, 50%, and 100% treatment groups (Figure 4A). The 25% treatment group had a single animal with elongated right and left gubernacular cords (>20 mm) and both testes were fluid-filled and atrophic. There was a large increase in the presence of malformations and anomalies in the 100% dose group, with 64.7% of animals displaying at least 1 reproductive tract malformation. Notably, there was a 47.1% incidence of hypospadias, 61.8% incidence of incomplete PPS, 11.8% incidence of cleft prepuce, 20.6% incidence of cleft phallus, and 8.8% incidence of exposed *os penis*. The male gonads in the 100% treatment group were impacted by an 11.7% incidence of testicular malformations (atrophic and/or fluid-filled), 5.9% incidence of epididymal agenesis, and 2.9% incidence of cryptorchidism. Males in the 100% dose group also displayed vaginal pouch formation, absence of vas deferens, absence of ventral prostate, and absence of seminal vesicles (each at 2.9% incidence), which were not

Table 2. Effects of *In Utero* Exposure (GD14-18) to the 18-Chemical Mixture of "Antiandrogens" on Male Rat Reproductive Tissues at Fetal, Perinatal, and Adult Timepoints

	Mixture dose					
	Control	6.25%	12.5%	25%	50%	100%
Fetal T prod (ng ml^{-1})[a]	9.42 ± 1.00	9.25 ± 0.90	8.49 ± 0.87	8.76 ± 0.49	**4.89 ± 0.80**	**2.69 ± 0.37**
AGD (mm)[b]	3.48 ± 0.15	3.23 ± 0.11	**3.15 ± 0.06**	**3.06 ± 0.05**	3.25 ± 0.09	**2.50 ± 0.08**
No. of nipples[c]	0.025 ± 0.025	0.43 ± 0.43	0.94 ± 0.34	**2.2 ± 0.8**	**2.2 ± 0.6**	**9.2 ± 0.7**
% with nipples[c]	2.5 ± 2.5	15.0 ± 15.0	**35.8 ± 0.1**	**54.4 ± 15.2**	**71.3 ± 14.4**	**100 ± 0**
Preputial separation (d)	43.4 ± 0.3	44.4 ± 0.8	44.8 ± 0.9	44.8 ± 0.7	44.3 ± 0.5	**47.0 ± 0.6**
Glans penis (mg)	128.1 ± 1.7	124.3 ± 1.8	**119.9 ± 2.4**	124.7 ± 3.9	126.2 ± 1.2	**110.1 ± 2.2**
Seminal vesicles (mg)	2021.3 ± 67.8	1879.5 ± 93.5	1897.0 ± 56.2	**1809.7 ± 56.1**	**1735.2 ± 62.8**	**1695.9 ± 24.3**
Paired testes (mg)	3994.9 ± 147.5	**3628.7 ± 40.4**	3786.4 ± 79.2	**3621.9 ± 136.5**	3816.3 ± 95.5	**3626.6 ± 71.3**
Paired epididymides (mg)	1474.1 ± 52.7	**1361.5 ± 46.2**	1391.8 ± 37.3	**1316.3 ± 36.5**	1397.9 ± 16.8	**1260.1 ± 32.5**
LABC (mg)	1623.7 ± 58.2	**1378.6 ± 56.2**	**1434.7 ± 40.3**	**1464.1 ± 14.0**	**1417.7 ± 52.0**	**1041.8 ± 68.0**
Adults w/nipples (%)	0 ± 0	0 ± 0	0 ± 0	**15.3 ± 7.2**	**13.3 ± 6.3**	**97.1 ± 2.9**
Hypospadias (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**47.1 ± 8.7**
Total malformed (%)	0 ± 0	0 ± 0	2.9 ± 2.9	**19.2 ± 7.9**	**13.3 ± 6.3**	**97.1 ± 2.9**

All values represent mean ± SEM, bold and gray are significantly different from controls ($p < .05$); all tissue weights from adults (≥PND120).
[a]*ex vivo* testis testosterone production (T prod) measured on GD18 following 3 h incubation.
[b]AGD measured on PND2.
[c]Nipple retention scored on PND13.

statistically significant; however, no control animals displayed any malformations.

Sperm was collected from the left epididymis and counted as 2 separate fractions, the corpus/caput and cauda (Supplementary Figure 1). Cauda sperm counts were significantly reduced in the 25%, 50%, and 100% treatment groups, corresponding to 18%, 17%, and 21% reductions compared with control, respectively. There was no effect of mixture exposure on corpus/caput sperm count, but due to the reductions in caudal sperm there was a significant overall decrease in total epididymal sperm in the 25% and 100% treatment groups (16% and 15% reductions, respectively; 12% reduction in the 50% dose group at $p = .065$).

Histopathological evaluation for each male included both testes and the right epididymis. The 100% treatment group displayed low grade inflammation of the testis ($n = 1$), and marked ($n = 1$) to severe ($n = 3$) testis atrophy/degeneration (Figure 5E); while the 25% treatment group displayed marked testis atrophy/degeneration ($n = 1$; Figure 5C) and moderate interstitial cell hyperplasia ($n = 1$; Figure 5C). Severe hypospermia of the epididymis was observed in the 25% ($n = 1$; Figure 5D) and 100% ($n = 2$) treatment groups, along with severe atrophy/degeneration of the epididymis ($n = 1$; Figure 5F) and an ectopic preputial gland ($n = 1$) in the 100% treatment. All treatment groups displayed low grade mononuclear cell infiltrate (lymphocytes and macrophages) in the epididymis with the highest incidence ($n = 7$) occurring in the lowest treatment (6.25%). No microscopic pathological findings were noted in the control group.

Maternal and Female Offspring Evaluation

There were no significant adverse effects on maternal health resulting from chemical exposure. Dams gained an average of 25.9 ± 6.5 g body weight during the dosing period and produced similarly sized litters of viable pups (13 ± 2 pups) as scored on PND2. Three dams displayed slightly delayed parturition of approximately 0.5 days (one 50% dam and two 100% dams), however by postcoital day 23 all dams had fully delivered. Dams averaged 14 ± 1 uterine implantation sites with 91% ± 10% pup viability to PND2 and 86% ± 12% pup viability to PND13. Female offspring displayed no differences in body weight or gross adult reproductive anatomy at necropsy.

DISCUSSION

Defects of the male reproductive tract seen in boys at birth are some of the most commonly occurring malformations in humans and these can be induced in laboratory animals by environmental chemicals and pharmaceuticals that disrupt androgen signaling. Our goal was to test whether a broad mixture of "antiandrogenic" chemicals would produce permanent defects in male rat reproductive tissues when each chemical in the mixture was below a dose that would produce any effect in an independent exposure. Severe effects including delayed puberty, hypospadias, cryptorchidism, weight reductions in nearly all androgen-dependent reproductive tissues, and reductions in sperm production occurred when each chemical was present at 5-fold below their respective LOAELs. At that dose each chemical was approximately 2-fold below their respective no observed adverse effect level (NOAEL; range, 0.5- to 5-fold) except for DDE and finasteride, which were both present at 2-fold greater than their NOAEL (Supplementary Table 3). However, even at the lowest dose tested (LOAEL/80), multiple male reproductive tissues (eg, glans penis, epididymis, testes, LABC) displayed significant weight reductions. At that dose, each chemical was, on average, approximately 34-fold (range, 8- to 80-fold) below its NOAEL for male reproductive tract alterations. Clearly, the chemicals tested here cumulatively produced adverse effects at doses well below their individual NOAELs, even though they disrupt the androgen signaling pathway during development via several different MIEs.

The combination of chemicals in the mixture was not intended to be "environmentally relevant" or represent "typical" human exposures. The human exposome is highly variable across spatial and temporal scales and our understanding of co-exposures is limited to the specific target analytes within a given biomonitoring effort. The Centers for Disease Control National Health and Nutrition Examination Survey is one of the most extensive contaminant biomonitoring efforts in the world and is currently limited to analyzing for 308 of the tens of

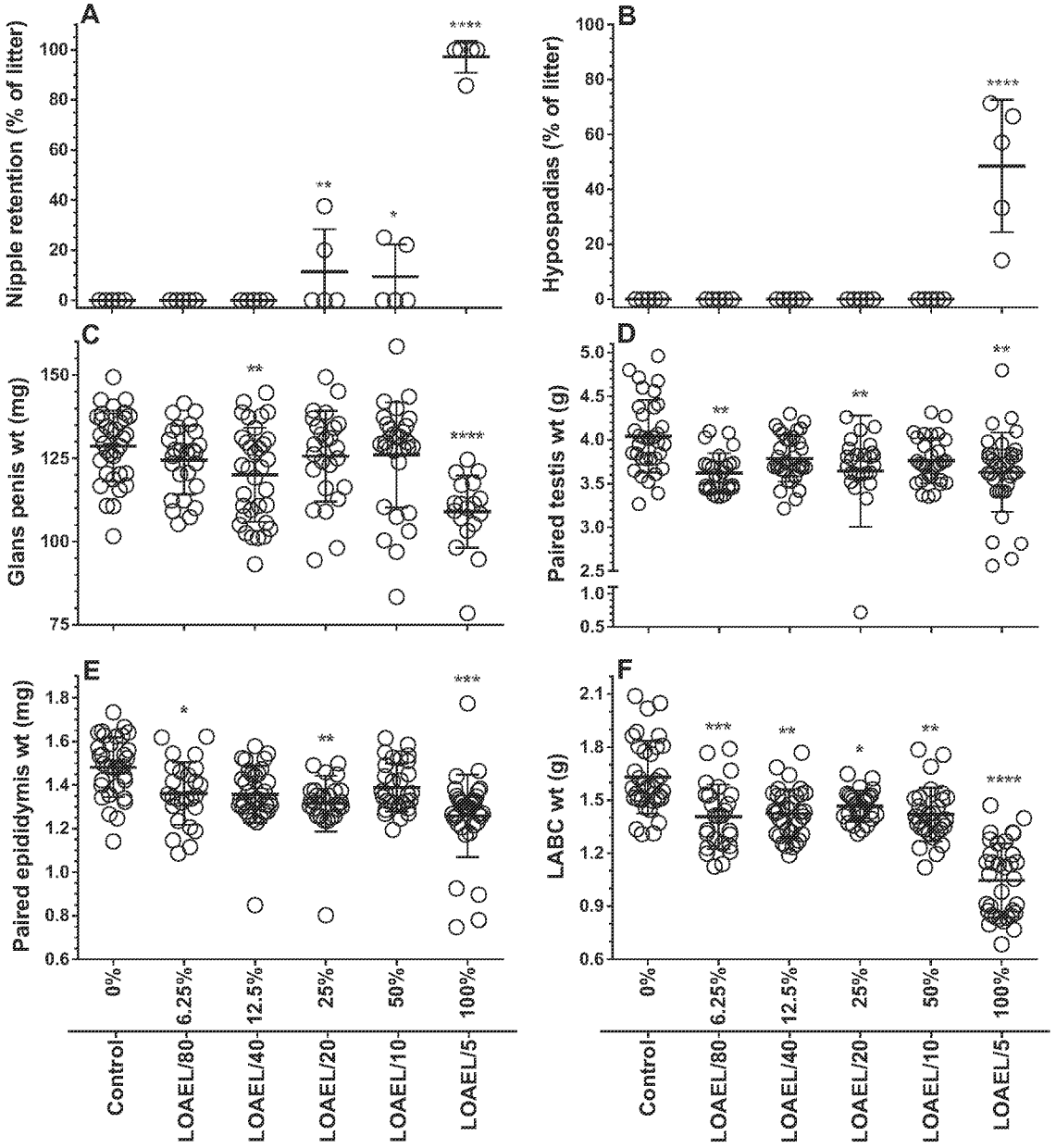

Figure 4. Treatment effects of *in utero* mixture exposure on adult male reproductive tract malformations (nipple retention, A; and hypospadias, B; displayed as mean % of litter affected) and weights of selected male reproductive tissues (glans penis, C; combined weight of testes, D; combined weight of epididymides, E; and LABC muscle complex, F). Tissue weight figures display all individual values within a treatment, however statistical analyses were conducted controlling for litter effects. Asterisks indicated significant difference as compared with control (****$p < .0001$, ***$p < .001$, **$p < .01$, *$p < .05$).

thousands of chemicals in industrial use (CDC, 2009). There are tendencies for some chemicals to co-occur within individuals (Kapraun *et al.*, 2017) and many of the persistent organic pollutants (including polychlorinated biphenyls, brominated flame retardants, polyfluorinated compounds, dioxins, and furans) and rapidly metabolized but ubiquitously occurring compounds (including bisphenols and phthalates) are nearly universally detected in all individuals (Dereumeaux *et al.*, 2016; Pumarega *et al.*, 2016; Qian *et al.*, 2015). However, considering that 3 of the compounds in the present mixture are prescription pharmaceuticals, the probability that a pregnant female would be coexposed to the specific mixture tested here is very low. Rather, the intention was to represent as many MIEs as possible that share a common downstream key event (KE; here, reduced AR-dependent mRNA/protein expression) and eventually produce common adverse outcomes (AO) at the whole organism level as a proof-of-concept for future mixture studies and cumulative risk assessment approaches.

Adverse outcome pathways (AOPs) provide a conceptual framework for grouping chemicals for mixture studies and conducting cumulative risk assessment (Ankley *et al.*, 2010). Thus far, grouping chemicals that are "toxicologically similar" has narrowly focused on specific chemical classes sharing a common specific MIE (eg, organophosphates). However, a narrow approach may exclude compounds that are biologically relevant from such groupings. As demonstrated here, AOPs that share

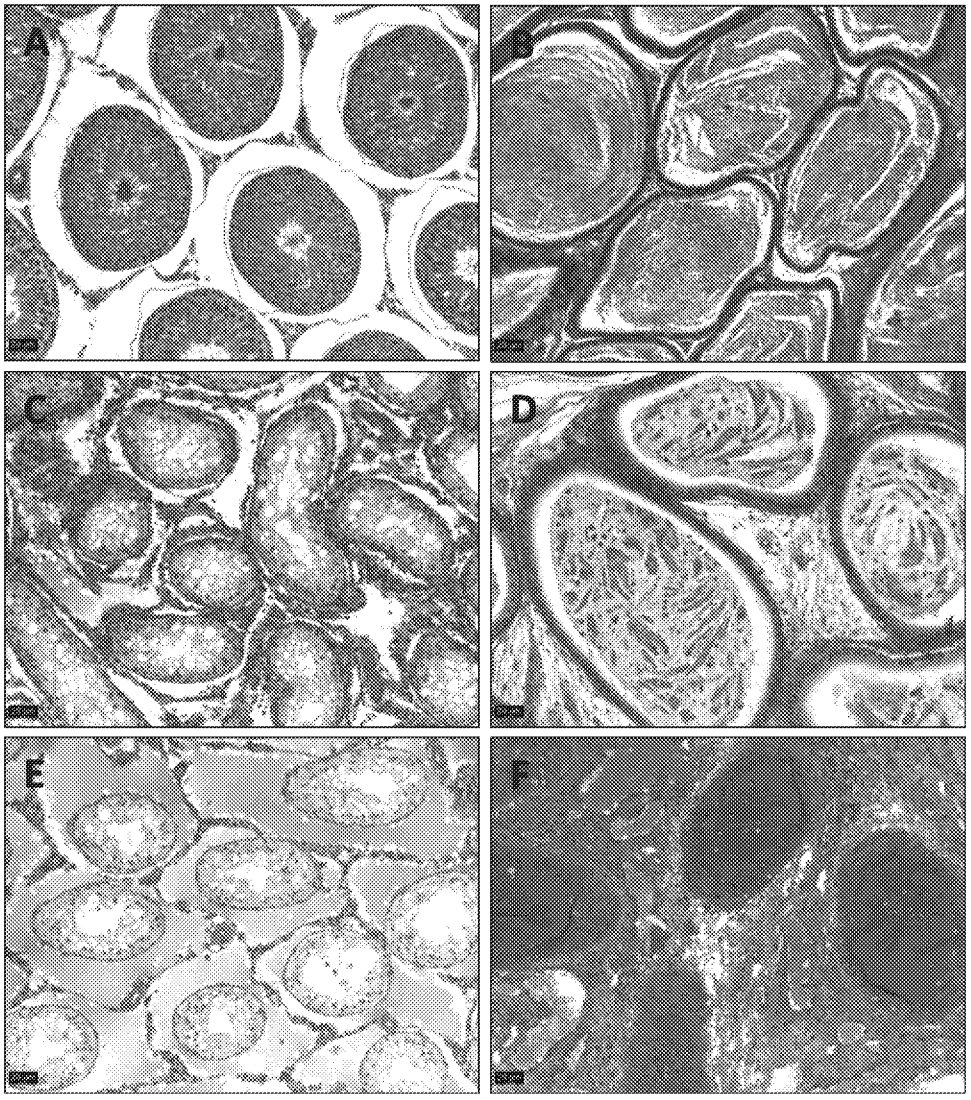

Figure 5. Hematoxylin and eosin staining of control testis (A) and epididymis (left cauda; B) from adult (>PND 120) male rats. *In utero* exposure to the 25% dose produced 1 animal with testis atrophy/degeneration and interstitial cell hyperplasia (C) and epididymis hypospermia (D). Males in the 100% dose displayed testis atrophy/degeneration ($n = 4$; E), hypospermia ($n = 2$, not shown), ectopic preputial gland ($n = 1$, not shown) and epididymis atrophy/degeneration ($n = 1$; F).

one or more critical KEs and/or the ultimate AOs can be combined into an AOP network that forms the basis for identifying chemicals that are toxicologically similar and adversely affect a common signaling pathway (Escher *et al.*, 2017). Moving forward, modern screening approaches can identify subsets of chemicals that target MIEs within an AOP network for assessment groupings. It is important to note that in this study, the short-term *in vivo* fetal testis screen is critical for identifying some compounds (eg, phthalates) that reduce fetal testis testosterone production but do not presently have an MIE identified that can be targeted using *in vitro* screening.

This study supports a substantial compilation of previous findings on the effects of *in utero* exposure to broad mixtures of "anti-androgens" (Table 3). Numerous prior experiments from our laboratory and others using binary and complex mixtures of chemicals with identical MIEs have shown cumulative, dose additive effects on male reproductive development (Christiansen *et al.*, 2008; Hannas *et al.*, 2011b; Hass *et al.*, 2007; Howdeshell *et al.*, 2007, 2008, 2015; Metzdorff *et al.*, 2007; Rider *et al.*, 2009). Additionally, there is a growing literature of experiments on cumulative, dose additive effects on male reproductive development from binary and complex mixture studies of chemicals with diverse MIEs that share a common KE in an "antiandrogen" AOP network (Axelstad *et al.*, 2014, 2018; Beverly *et al.*, 2014; Christiansen *et al.*, 2009, 2012; Hass *et al.*, 2012; Hotchkiss *et al.*, 2004, 2010; Isling *et al.*, 2014; Jacobsen *et al.*, 2010; Rider *et al.*, 2008, 2010; Schneider *et al.*, 2017). The majority of these studies have been conducted in our laboratory (L.E. Gray, USEPA) and the laboratory of Ulla Hass (Technical University of Denmark). In experiments from our laboratory testing complex "antiandrogen" mixtures similar to the present study, Rider *et al.* (2008, 2010) tested a 7-chemical mixture (vinclozolin, procymidone, linuron, prochloraz and benzyl butyl, dibutyl, and diethyl hexyl phthalates) and a 10-chemical mixture (7 chemical mixture plus diisobutyl, diisoheptyl, and dipentyl phthalate), respectively. In both studies, dose additive effects on male reproductive tract development were observed when each chemical in the mixture was present at dose levels near the individual chemical NOAELs. In this study, we expanded the number of chemicals and range of MIEs in the mixture. As a result, we

Table 3. Compilation of Mixture Studies Reporting Cumulative Adverse Effects From *In Utero* Exposure to Chemicals Disrupting Androgen-Dependent Tissue Development in the Rat Via Similar and Diverse Mechanisms of Action Within the AR Signaling Pathway

Type of Mixture	Chemicals in Mixture	Chemical Mechanisms	References
Similar mechanism	DBP + DEHP	Phthalate mechanism	Howdeshell *et al.* (2007)
	PCD + VIN	AR antagonist	Howdeshell *et al.* (2008), Rider *et al.* (2009)
	BBP + DBP	Phthalate mechanism	Howdeshell *et al.* (2008), Rider *et al.* (2009)
	FLU + PCD + VIN	AR antagonist	Christiansen *et al.* (2008), Hass *et al.* (2007) Metzdorff *et al.* (2007)
	BBP + DBP + DEHP + DIBP + DPEP	Phthalate mechanism	Howdeshell *et al.* (2008, 2015)
	BBP + DBP + DCHP + DEHP + DHEP + DHP + DIBP + DIHEP + DPEP	Phthalate mechanism	Hannas *et al.* (2011b)
Diverse mechanisms	DBP + PCD	Phthalate mechanism (DBP) + AR antagonist (PCD)	Hotchkiss *et al.* (2010)
	BBP + LIN	Phthalate mechanism (BBP) + Mixed mechanism[a] (LIN)	Hotchkiss *et al.* (2004)
	DPEP + SMV	Phthalate mechanism (DPEP) + Cholesterol inhibitor (SMV)	Beverly *et al.* (2014)
	FLU + PCZ + VIN	AR antagonist (FLU, VIN) + Mixed mechanism (PCZ)	Schneider *et al.* (2017)
	DEHP + FIN + PCZ + VIN	Phthalate mechanism (DEHP) + 5α-reductase inhibitor (FIN) + Mixed mechanism (PCZ) + AR antagonist (VIN)	Christiansen *et al.* (2009)
	ECZ + MCZ + PCD + PCZ + TCZ	AR antagonist (MCZ, PCD) + Mixed mechanism (ECZ, PCZ, TCZ)	Hass *et al.* (2012), Jacobsen *et al.* (2010)
	BBP + DBP + DEHP + LIN + PCD + PCZ + VIN	Phthalate mechanism (BBP, DBP, DEHP) + Mixed mechanism (LIN, PCZ) + AR antagonist (PCD, VIN)	Rider *et al.* (2008)
	DBP + DDE + DEHP + ECZ + PCD + PCZ + LIN + VIN	Phthalate mechanism (DBP, DEHP) + AR antagonist (DDE, PCD, VIN) + Mixed mechanism (ECZ, LIN, PCZ)	Axelstad *et al.* (2014, 2018), Christiansen *et al.* (2012); Isling *et al.* (2014)
	BBP + DBP + DEHP + DIBP + DIHEP + DPEP + LIN + PCD + PCZ + VIN	Phthalate mechanism (DBP, BBP, DEHP, DiHP, DPeP, DiBP) + Mixed mechanism (LIN, PCZ) + AR antagonist (PCD, VIN)	Rider *et al.* (2010)
	BBP + DBP + DCHP + DDE + DEHP + DHEP + DHP + DIBP + DIHEP + DPEP + FIN + FLU + LIN + PCD + PCZ + PFQ + SMV + VIN	Phthalate mechanism (DBP, BBP, DEHP, DiHP, DPeP, DiBP, DCHP, DHP, DHeP, DiHP) + AR antagonist (DDE, FLU, PCD, PFQ, VIN) + 5α-reductase inhibitor (FIN) + Mixed mechanism (LIN, PCZ) + Cholesterol inhibitor (SMV)	Present study

Adapted from Howdeshell *et al.* (2017).

[a]Mixed mechanism indicates chemicals known to be both AR antagonists and direct inhibitors of CYP steroidogenic enzymes and reduce fetal testosterone production.

BBP, benzyl butyl phthalate; DBP, dibutyl phthalate; DCHP, dicyclohexyl phthalate; DDE, 1, 1'-(2, 2-dichloro-1, 1ethenediyl)bis(4-chlorobenzene); DEHP, bis(2-ethylhexyl) phthalate; DHEP, diheptyl phthalate; DHP, dihexyl phthalate; DIBP, diisobutyl phthalate; DIHEP, bis(5-methylhexyl) phthalate; DPEP, dipentyl phthalate; ECZ, epoxiconazole; FIN, finasteride; FLU, flutamide; LIN, linuron; MCZ, mancozeb; PCD, procymidone; PCZ, prochloraz; PFQ, pyrifluquinazon; SMV, simvastatin; TCZ, tebuconazole; VIN, vinclozolin.

observed significant effects on male offspring at 122-fold (range, 24- to 545-fold) and 47-fold (range, 10- to 218-fold) lower doses for the individual chemicals than did Rider *et al.* in the 7- and 10-chemical mixtures, respectively. Similarly, the Hass lab has tested a 4 chemical mixture (diethylhexyl phthalate, finasteride, prochloraz, vinclozolin) with effects occurring at 32-fold (range, 11- to 67-fold) greater doses than the present study (Christiansen *et al.*, 2009). Additional studies by their group examined a mixture of 8 "antiandrogens" (dibutyl phthalate, p, p'-DDE, diethylhexyl phthalate, epoxiconazole, procymidone, prochloraz, linuron, and vinclozolin) and a 13-chemical mixture that included the previous 8 chemical mixture plus 4 estrogenic compounds and paracetamol (Axelstad *et al.*, 2014, 2018; Christiansen *et al.*, 2012; Isling *et al.*, 2014). In those studies the 8 "antiandrogenic" compounds were the predominant drivers of male reproductive effects as compared with the full 13 chemical mixture with estrogenic compounds and paracetamol added. Again, the greater complexity of the present mixture resulted in

adverse effects at lower individual chemical doses for dibutyl phthalate, diethylhexyl phthalate, procymidone, prochloraz, and vinclozolin (12-fold [range, 1.2- to 29-fold]), however DDE and linuron were 8.3- and 1.7-fold lower, respectively, in the 8 chemical mixture from the Hass studies. Finally, Schneider *et al.* (2017) recently reported on a 3-chemical mixture (flutamide, prochloraz, and vinclozolin), with cumulative effects observed at a similar dose of flutamide used here, but 11- and 53-fold greater doses of prochloraz and vinclozolin, respectively. Overall, numerous laboratory studies have now demonstrated that *in utero* exposure to chemicals that disrupt androgen signaling via diverse mechanisms of toxicity produce cumulative effects on male reproductive tract development and that the doses of individual chemicals needed to produce effects generally appear to be inversely related to the number of chemicals in the mixture.

Although not a primary goal of the present study, we were interested in the accuracy of component-based statistical models for estimating cumulative effects of antiandrogenic mixtures. We calculated dose addition and response addition model predictions for mixture effects on AGD at PND2 (Supplementary Figure 2). The ED_{50} calculated from the observed data was beyond the tested dose range (164% of top dose), therefore we calculated an ED_{90} and ED_{60} to compare to the observed data. Response addition estimated an ED_{90} (9.6% of top dose) that was slightly more accurate than dose addition (27.7%) when compared with the observed data (95% CI, 1.3%–24.2%). However, for the ED_{60} calculation both response addition (120.7%) and dose addition (104.2%) produced estimates within the 95% CI of the observed data (62.2%–314.7%). Overall, the dose addition modeling approach has received broad support as the most appropriate default model for estimating mixture effects of antiandrogens in a component-based approach (Christiansen *et al.*, 2009; European Commission, 2012; Hass *et al.*, 2012; NRC, 2008; Rider *et al.*, 2008, 2010).

To our knowledge, this study represents one of the most complex *in vivo* mixtures studies conducted to date, and the most complex incorporating *in utero* exposure. In addition to the previously mentioned mixture studies from the Hass group, Wade *et al.* (2002) exposed mature male rats to a mixture of 18 organochlorines and metals that are commonly detected in human tissues with a dosing structure based on chemical NOAELs. Following 70 days of exposure, the authors reported only minor reproductive effects at individual chemical concentrations 10-fold greater than the NOAELs and above. Crofton *et al.* (2005) exposed female rats to an 18-chemical mixture consisting of polyhalogenated aromatic compounds (ie, dioxins, furans, PCBs) and reported slightly greater than additive effects (2- to 3-fold) on reductions in serum thyroid hormone concentrations. Importantly, even though those studies and this study represent the most complex mixtures toxicology research conducted to date, they still under-represent the breadth of chemical exposures/residues that humans typically experience (Pumarega *et al.*, 2016). As demonstrated here, LOAELs determined on an individual chemical basis may under-predict the toxicity to the unborn fetus given the high likelihood of multiple chemical coexposures.

For risk assessment to fully protect human health it is critically important to consider the potential hazard to the unborn child from multiple chemical exposures together, not just one chemical at a time. Our research, and the research of others, has provided evidence that *in utero* exposure to antiandrogenic chemicals produces cumulative effects on male reproductive development and that these effects can be relatively accurately predicted using dose addition modeling. Given the principles of dose addition, one could assume that the greater the number of antiandrogens in an exposure the lower the dose of any one chemical is necessary to contribute towards additive effects. The results of the present study support this assumption in that the mixture tested was more complex and adverse effects were observed at lower individual chemical doses than any other previously published study taking a similar approach. This relationship holds despite the component chemicals targeting different molecular mechanisms. Adverse effects can occur at exposure levels considerably below individual chemical effect levels (ie, LOAEL, NOAEL) when mixed with additional compounds that impact the same biological signaling pathway. This study suggests that male fetuses may be at increased risk due to cumulative exposure of the pregnant woman to multiple "antiandrogenic" chemicals, even when individual doses are below known levels of concern. More work is needed to identify the breadth and depth of human exposures to mixtures of environmental chemicals that may interact in concert at critical developmental periods to produce adverse effects.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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